IN VITRO EVALUATION OF CANDIDATE PRETREATMENT AND TREATMENT COMPOUNDS AGAINST SULFUR MUSTARD (HD)-INDUCED HUMAN MONONUCLEAR LEUKOCYTE TOXICITY USING A DYE EXCLUSION CELL VIABILITY ASSAY

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ABSTRACT

An assay measuring propidium iodide (PI) incorporation into nonviable human peripheral blood mononuclear leukocytes (PBML) was established at the U.S. Army Medical Research Institute of Chemical Defense (USAMRICD), and the technology transferred and implemented at Battelle's Medical Research and Evaluation Facility (MREF) for use as a screen to evaluate candidate compounds for direct cytotoxicity as well as for efficacy in preventing HD-induced cytotoxicity. For assay transition, studies were performed to establish a fixed HD challenge concentration; to develop a positive and negative control dataset; and to establish the reproducibility in obtaining an EC_{50} (concentration of candidate compound required to provide 50 percent protection against the fixed HD concentration) for niacinamide (NM). Various concentrations of candidate compounds were preincubated for 15 to 30 min with PBML prior to adding the fixed HD challenge. At 24 hr after exposure, PI was added to the cultures and the number of nonviable (PI positive) cells was determined by flow cytometry. Positive (NM pretreated) and negative (HD only) controls were examined concurrently and used to maintain data quality. From this dataset, candidate compounds were evaluated for direct cytotoxic effects and for efficacy in preventing HD-induced cytoxicity. EC_{50} values for effective candidate compounds were estimated and reported for ranking compound effectiveness. Results from these studies demonstrate assay function and reproducibility during routine screening operations.

This work was supported by the U.S. Army Medical Research and Development Command under Contract DAMD17-89-C-9050.

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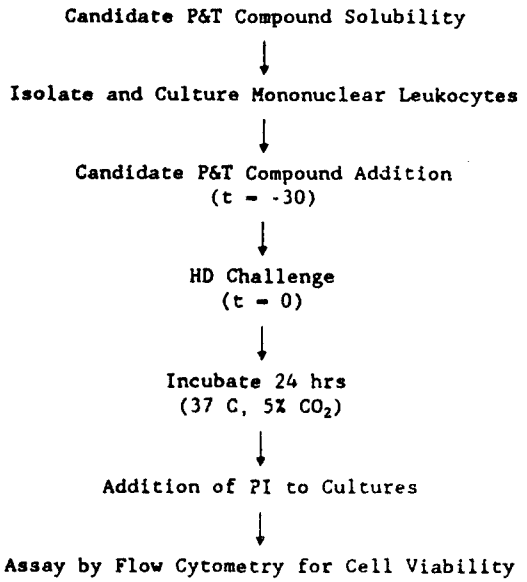
INTRODUCTION

2,2'-dichloroethyl sulfide (mustard, HD) is a powerful alkylating agent that causes incapacitating injuries to the eyes, respiratory tract and skin. HD is known to irreversibly bind cellular DNA, RNA, and proteins[1], however the precise biochemical mechanism of HD-induced vesication is not known. The Drug Assessment Division of USAMRICD has the mission of evaluating candidate pretreatment and therapeutic (P&T) compounds and identifying those which are safe and effective in preventing or reversing the effects of chemical warfare agents such as HD. Our laboratory, in conjunction with USAMRICD, has established a routine in vitro screen to analyze the effectiveness of candidate P&T compounds against HD-induced cytotoxicity. This screen has provided a mechanism to rank order the effective P&T compounds as well as provide information about the pathogenesis following exposure to HD.

MATERIALS AND METHODS

HD, along with data regarding its purity, was supplied by the U.S. Army. In accordance with procedures approved by Battelle's Human Subjects Committee, blood samples were collected from healthy human volunteers and mononuclear leukocytes were isolated by density (Percoll 1.080 gm/mL) centrifugation. Preliminary studies were performed to verify the flow cytometer settings. Fluorescent-labelled antibody specific for a white blood cell surface antigen (CD45 antigen) was used to confirm the forward scatter or particle size threshold setting for the isolated leukocytes. For compound evaluations, candidate P&T compounds were prepared at a stock solution concentration of 2 mM (pH 7.4). If the candidate compound remained insoluble after vortexing and sonicating, the pH of the diluent (RPMI 1640) was lowered to 7. If the compound remained insoluble at pH 7, the stock solution concentration was lowered to 0.2 mM. If after vortexing and sonicating, it still remained insoluble, the compound was not analyzed. The cell system consisted of one million cells in RPMI 1640 in a total volume of 200 µL per well of a 96-well microplate. For compound evaluations, either control treatment compound (NM), candidate P&T compound, or vehicle (RPMI 1640) were added to cultures 15 to 30 min prior to HD. After a 24 hr incubation at 37 C in a 5 percent carbon dioxide, water saturated atmosphere, 50 µL of a 300 µg/mL propidium iodide solution was added, and the number of propidium iodide positive cells relative to the total (10,000 cells) examined was determined using a Becton Dickinson FACScan® flow cytometer. An HD concentration response study was initially performed to establish a fixed HD challenge concentration for use in subsequent studies to assess assay reproducibility, to establish assay control database values, and to evaluate candidate P&T compound efficacy. Shown below is a flowchart of procedures used for evaluating candidate P&T compounds through the cell viability assay:



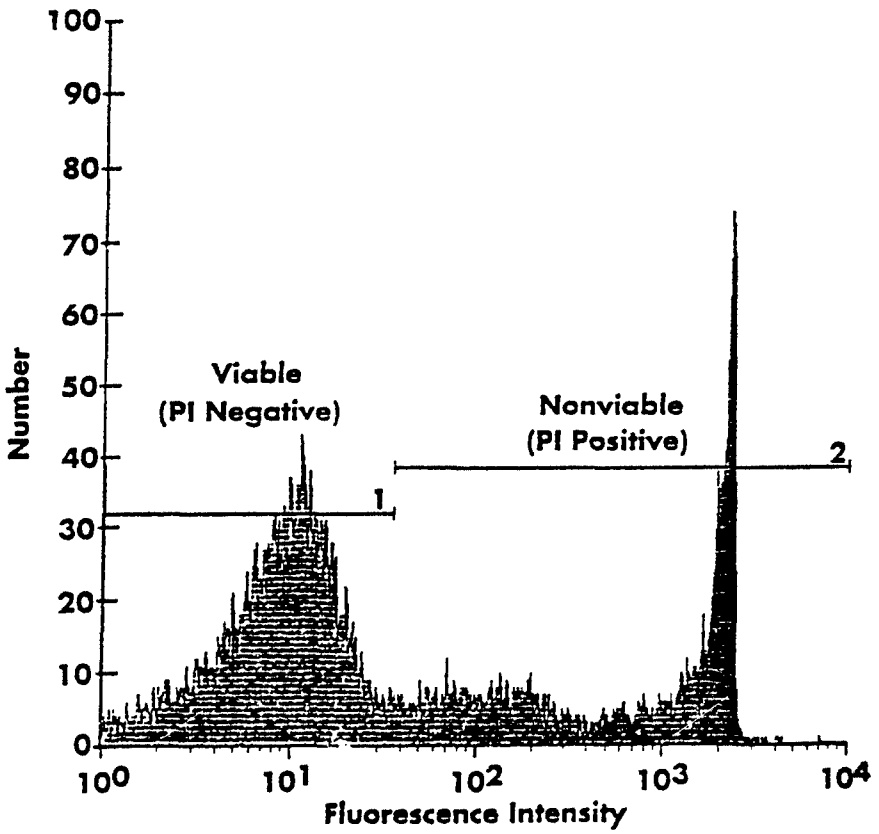
The treatment groups used for efficacy and variability evaluations were as follows:

- Cells received no HD and no candidate P&T compound (Vehicle Control),
- Cells were pretreated with at least five concentrations of candidate P&T compound and not challenged with HD Evaluate Direct Cytotoxicity),
- Cells received the HD MR_{87} (the HD concentration required to produce 87 percent of the maximal response), (Negative Control),
- Cells were pretreated with 1 mM NM and then challenged with the HD MR_{87} (Positive Control),
- Cells were pretreated with at least five different concentrations of the candidate P&T compound and then challenged with the HD MR_{87} (Efficacy Evaluation), and
- Cells were challenged with the HD MR_{100} (the HD concentration that should produce maximal cytotoxicity response).

RESULTS

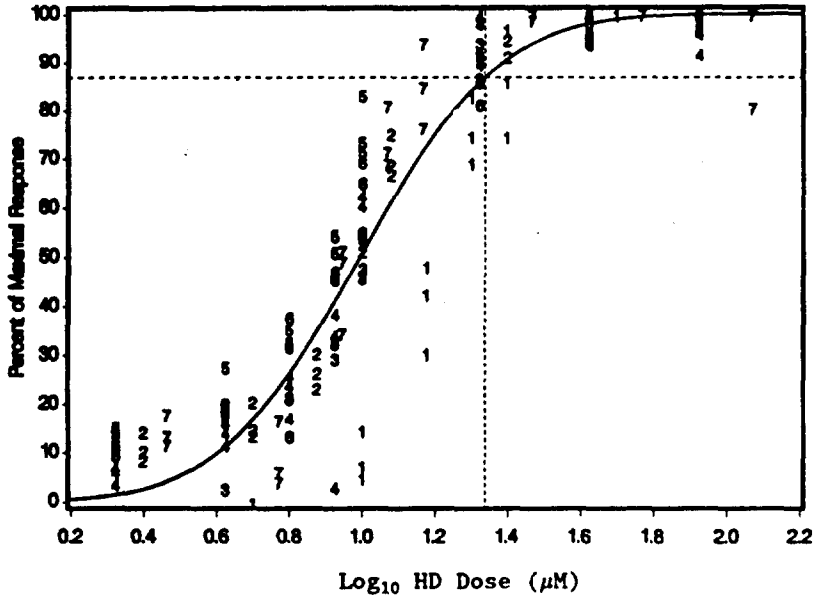
The flow cytometer demonstrated that 95 percent of the particles from which data were being collected stained positive with the anti-CD45 antibody. For all cytotoxicity assays, vehicle and HD exposed samples were included for use in optimizing fluorescent detector parameter voltages. Concentration-response datasets for HD-induced cytotoxicity were obtained for mononuclear cells isolated from seven donors. The viability data were standardized for both the level of natural cell death observed in the vehicle control samples and the maximal amount of HD-induced cytotoxicity for each donor, and expressed as percent maximal cytotoxicity. The standardized percent maximal response data from the seven donors were combined and the HD MR_{87} was estimated to be 21.8 μM (95 percent confidence limits of 19.5 to 25.0 μM). Shown in Figure 1 is a flow cytometer histogram for HD-exposed cells which illustrates the fluorescent discrimination of viable and nonviable cells.

FIGURE 1. HISTOGRAM OF PROPIDIUM IODIDE (PI) FLUORESCENCE INTENSITY FOR AN HD-EXPOSED SAMPLE



The log HD concentration response relationship established for the seven donors is shown in Figure 2. The PBML from donor one appears to have responded somewhat differently than the PBML's from the other six donors. As no experimental reason for omitting the data existed, the data was retained.

FIGURE 2. HD CONCENTRATION RESPONSE FOR MONONUCLEAR LEUKOCYTE CYTOTOXICITY



The HD MR_{87} was used as the fixed HD challenge concentration in subsequent studies to establish initial negative (HD only) and positive (HD, 1 mM NM pretreated) control group values as well as for test compound evaluations. Data were initially obtained for the positive and negative control groups and the assay control limits (three standard deviations) were established. The control limits for the negative control ranged from 68.3 to 99 percent of the maximal cytotoxic response, and the control limits for the positive control from 70.2 to 101.6 percent protection from HD-induced cytotoxicity. These controls were included in all test compound evaluation studies, and the database was periodically examined and updated.

As the test compound EC_{50} estimates are to be used for ranking compound effectiveness, the reproducibility in obtaining the EC_{50} estimate was determined for the positive control compound NM by examining day-to-day and donor-to-donor effects. Initial evaluations indicated little day-to-day effect for a given donor (C.V. = 1.8 percent) and slightly more effect among donors evaluated on separate days of testing (C.V. = 7.0 percent).

As part of the assay control process, negative and positive controls are included with each experimental evaluation and the EC_{50} for NM is periodically determined. Shown in Figure 3 are the NM EC_{50} values determined over time for an expanded donor pool. Use of an expanded donor pool (11 individuals) provided a better estimate of the average NM EC_{50} (38.4 μM) and the variability (SD = 6.1) associated with this estimate. For this dataset, the C.V. was 16 percent. As an additional level of control, the mononuclear leukocytes from several donors are being tracked and have been found to provide relatively consistent results over time.

FIGURE 3. CONTROL CHARTING OF NIACINAMIDE EC_{50} ESTIMATES

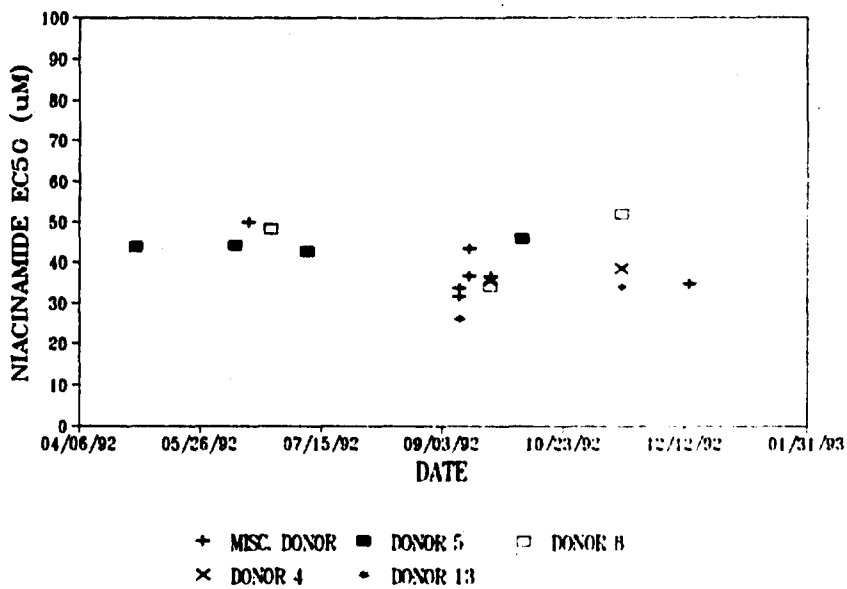


Table 1 is a summary of the results from efficacy assessment studies for 37 candidate P&T compounds. The candidate compounds were evaluated for direct cytotoxicity and for effectiveness against HD-induced cytotoxicity. Two candidate P&T compounds could not be analyzed due to solubility problems. Eighteen of the 35 compounds were found to be more effective ($p \leq 0.05$) than the negative (MR_{87}) control. Twelve of the 18 effective compounds produced EC_{50} estimates ranging from 180.4 to 3.3 μM.

TABLE 1. EFFECTIVENESS OF USAMRICD CANDIDATE P&T COMPOUNDS

Candidate Compound	NOEL[a]	MEC[b] (μM)	EC_{50}[c] (μM)	95% Confidence Limits Lower	Upper	Slope	(SE)
ICD-1841	N/D[d]	N/D	N/D	N/D	N/D	N/D	N/D
ICD-2064	N/D[d]	N/D	N/D	N/D	N/D	N/D	N/D
ICD-1365	100[e]	N/E[g]	N/E	N/E	N/E	N/E	N/E
ICD-1447	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1453	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1457	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1551	100[e]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1570	30[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1594	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1671	0.1[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1704	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1770	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1773	30[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1779	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1824	1000	N/E	N/E	N/E	N/E	N/E	N/E
ICD-1993	30[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-2083	100[e]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-2087	1[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-2525	300[f]	N/E	N/E	N/E	N/E	N/E	N/E
ICD-0955	1000	1000	N/C[h]	N/C	N/C	N/C	N/C
ICD-0037	1000	300	N/C	N/C	N/C	N/C	N/C
ICD-2153	300[e]	300	N/C	N/C	N/C	N/C	N/C
ICD-1316	100[e]	100	N/C	N/C	N/C	N/C	N/C
ICD-1541	1000	3	N/C	N/C	N/C	N/C	N/C
ICD-2151	1000	3	N/C	N/C	N/C	N/C	N/C
ICD-0968	1000	30	180.4	169.7	191.8	1.76	(0.10)
ICD-2065	1000	10	68.6	58.0	81.2	1.96	(0.21)
ICD-0964	1000	10	64.4	60.4	68.8	2.16	(0.10)
ICD-1478	100[e]	30	56.3	52.0	61.3	2.57	(0.89)
ICD-1797	1000	10	52.7	46.7	59.5	1.60	(0.12)
ICD-0967	1000	10	43.8	40.5	47.4	2.51	(0.17)
ICD-1446	1000	30	23.9	19.4	29.5	3.70	(0.77)
ICD-2066	100[e]	3	16.3	13.8	19.2	1.77	(0.16)
ICD-2062	1000	3	9.9	7.8	12.5	1.17	(0.09)
ICD-1794	300[e]	3	9.3	8.2	10.6	1.54	(0.12)
ICD-2063	100[e]	3	4.6	3.8	4.4	5.00	(0.48)
ICD-2163	1000	3	3.3	2.8	3.8	4.47	(3.31)

[a]NOEL - No observable effect level.
[b]MEC - Minimum effective concentration.
[c]EC_{50} - Candidate compound concentration estimated to provide 50 percent protection against HD-induced cytotoxicity.
[d]N/D - Not determined due to compound solubility problems
[e]Highest concentration evaluated due to solubility.
[f]Highest concentration evaluated due to direct cytotoxicity.
[g]N/E - Not effective; no statistical difference ($p \leq 0.05$) from the negative (MR_{87}) control.
[h]N/C - EC_{50} could not be estimated due to partial effectiveness at concentration tested.

CONCLUSIONS

- The assay has operated in a reproducible fashion over the duration of the candidate P&T compound screening effort.
- The viability assay measuring PI uptake to nonviable human PBML as a measurement of efficacy against HD cytotoxicity is a dependable screen and provides a good index for rank ordering the effectiveness of compounds against HD-induced cytotoxicity.

ACKNOWLEDGMENTS

The authors wish to acknowledge Rebecca Geer, Carole Wickline, Jean Truxall, and Jack Waugh for their technical assistance in these studies.

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[1] Calabresi, P. and B.A. Chabner, "Antineoplastic Agents" in Goodman and Gilman's The Pharmacological Basis of Therapeutics, Eighth Edition", A.G. Gilman, T.W. Rall, A.S. Nies, and P. Taylor, eds., Pergamon Press, New York, 1209-1263, (1990).